Journal of Chemical Ecology Vol. 32, No. 3, March 2006 (©2006)
DOI: 10.1007/s10886-005-9026-3

DIMETHYLFURAN-LACTONE PHEROMONE FROM MALES OF *Galerucella calmariensis* AND *Galerucella pusilla*·

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(Received June 23, 2005; revised October 13, 2005; accepted October 24, 2005)

Published Online April 4, 2006

Abstract—Male *Galerucella calmariensis* and *Galerucella pusilla* (Coleoptera: Chrysomelidae) emit an aggregation pheromone while feeding on host foliage. Isolation of the compound from collected volatiles was guided by comparisons of gas chromatograms of extracts from males and females and by gas chromatography–electroantennographic detection. The compound was identified by a combination of spectrometric methods and microchemical tests as the novel dimethylfuran lactone, 12,13-dimethyl-5,14-dioxabicyclo[9.2.1]-tetradeca-1(13),11-dien-4-one. The structure was confirmed by synthesis, and the synthetic compound attracted males and females of both species in field bioassays. These beetles were previously introduced into North America as biological control agents for the invasive wetland weed, purple loosestrife *Lythrum salicaria*, and the pheromone could become a tool for monitoring populations. A new method is described for distinguishing the two species based on the tibial spurs of the males.

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Key Words—*Galerucella calmariensis*, *Galerucella pusilla*, Coleoptera, Chrysomelidae, purple loosestrife, *Lythrum salicaria*, biological control, lactone, synthesis, attraction, aggregation pheromone.

INTRODUCTION

Galerucella calmariensis (L.) and *Galerucella pusilla* (Duftschmidt) are leaf beetles (Coleoptera: Chrysomelidae) that were introduced into the United States from Europe as biological control agents for the invasive wetland weed, purple loosestrife *Lythrum salicaria* L. (Hight et al., 1995). The adults are small (3.5–5.5 mm) and brownish with dark markings; the species and sexes are nearly identical in appearance. The insects consume the foliage of purple loosestrife, both as adults and as larvae. For biocontrol purposes, methods of monitoring release sites for beetle establishment, abundance, seasonal timing, dispersal rates, and other population properties are required. Pheromone-baited traps could be sensitive and selective tools for acquiring such information. Observations of aggregation behavior in these beetles suggested that a pheromone exists (Grevstad and Herzig, 1997), but none had been chemically identified.

Existing knowledge about chrysomelid pheromones gave little guidance on *Galerucella* spp. because of the considerable chemical and biological diversity within the family. Pheromones of the rootworm beetles (*Diabrotica* spp.) are female produced and attract only males; they consist of esters of methyl-branched secondary alcohols and structurally related ketones (reviewed in Krysan et al., 1989). The more recently identified chrysomelid pheromones are all male produced and attract both sexes. These include a hydroxyketone from the cereal leaf beetle *Oulema melanopus* L. (Cossé et al., 2002; Rao et al., 2003), a dihydroxyketone from the Colorado potato beetle *Leptinotarsa decemlineata* Say (Dickens et al., 2002), a series of himachalene and cadinene sesquiterpenes from *Aphthona* and *Phyllotreta* flea beetles (Bartelt et al., 2001, 2003; Muto et al., 2004; Soroka et al., 2005), and a 7-carbon diene aldehyde and alcohol from *Diorhabda elongata* Brullé (Cossé et al., 2005).

An approach was chosen that would detect pheromone emission by either sex. Volatiles were collected from male and female beetles separately and analyzed for sex-specific compounds that elicited strong antennal responses in gas chromatography–electroantennographic detection (GC–EAD) studies as possible pheromone components. Only one such compound was found from *G. calmariensis* (from males), and it was subsequently found from *G. pusilla* males also. We report here the identification, synthesis, and field testing of the pheromone, which was attractive to both sexes of each species. Structurally, the compound is unlike any of the previously identified chrysomelid pheromones.

METHODS AND MATERIALS

Insects. G. calmariensis were reared on potted purple loosestrife plants in a greenhouse at the Illinois Natural History Survey. Adults for volatile-collection efforts in early spring were obtained from a stock of beetles that had diapaused in cold storage over the winter, and in late spring or summer, adults were collected as they emerged from pupation in the soil. The sexes of *G. calmariensis* were separated based on the characteristic tibial spurs on the meso- and metathoracic legs of males (Cossé, 2004). Early in the project, when it was unknown which sex emitted a long-range pheromone, adults were placed individually into vials as they emerged from overwintering diapause or from pupation to increase the likelihood that they were unmated when used for collection of volatiles. This precaution was taken because in another chrysomelid, *Diabrotica virgifera*, emission of the female-produced sex pheromone ceased upon mating (Bartelt and Chiang, 1977). Once evidence emerged for a male-produced pheromone in *G. calmariensis,* the time-consuming procedure of isolating beetles as they emerged was discontinued. In chrysomelids with male-produced pheromones (e.g., *Phyllotreta cruciferae*, *Aphthona* spp., and *Diorhabda elongata*), pheromones were collected from freely mating, field-collected males (Bartelt et al., 2001; Cossé et al., 2005).

During summer of 2003, *G. pusilla* became available from a population in Minnesota, allowing collection of volatiles and electrophysiology to be conducted for this species also. *G. pusilla* were also studied under the microscope for sexually dimorphic characters and differences from *G. calmariensis* that would allow identifications to be made without injuring the beetles. Additional field-collected *G. pusilla* were obtained during summer of 2005 for volatile collections.

Collection of Volatiles. Volatiles were collected from *G. calmariensis* during 2002–2004 at the National Center for Agricultural Utilization Research (NCAUR). Initial collections were made from both males and females feeding on purple loosestrife foliage and from foliage alone. Once it became clear that a male-produced pheromone was probable, subsequent collections were made almost entirely from males, accumulating material for identification. In 2002, 122 1- to 3-d collections were made from feeding males, 60 were made from feeding females, and 8 were made from plants only. In 2003, 567 1-d collections were made from males, and 23 from females. In 2004, 117 1-d collections were made, all from males. For *G. pusilla*, 50 1-d collections from males and 21 from females were made during 2003, and 100 1-d collections from males and 40 from females were made during 2005.

The conditions and apparatus for collecting beetle-produced volatiles evolved during the course of this study. Details for the most successful procedure (used in 2004 for *G. calmariensis*) are described here: The body of

the collector apparatus was a 45-cm-long by 3-cm-diameter straight glass tube with a female 24/40 ground glass joint on each end. A glass adapter was fitted into each end. (These 9-cm-long adaptors, suitable for holding thermometers or objects of similar diameter, have a male 24/40 joint on one end and a threaded fitting with an airtight O-ring seal on the other, Ace Glass, Vineland, NJ, USA #5028-30). Glass filter tubes (6 mm O.D. by ca. 8 cm length) containing a 1-cm plug of Super-Q were prepared as described previously (Cossé et al., 2002) and inserted into each adapter.

Each aeration chamber contained a shoot of purple loosestrife foliage about 30 cm in length after the growing tip was removed, obtained from potted plants maintained in the NCAUR greenhouse. Leaves were detached from the shoot until only three or four remained, each about 5–8 cm in length, well spaced along the stem. The base of the stem was placed in a 5-ml glass vial containing water. A Teflon seal in the ring-shaped cap of the vial kept the water from spilling. Beetles (usually 5–10) were added to the collector, which was then oriented horizontally. This configuration provided ample, but not excessive, food for the beetles for 1 d; using only this amount of foliage helped minimize the background levels of plant-related chemicals in the volatile collections. The beetles were not overcrowded and were able to move about on the plants in a natural manner.

Clean air was pulled through the aeration chamber and volatile trap at 300 ml/min. This rate was rapid enough that transpired water from the leaves did not condense on the walls of the tubes (the target compound was almost never detected when water droplets were visible). An inlet filter cleaned the entering air, and the outlet filter captured the volatiles emitted by the insects and plants. The collectors were located in an environmental chamber at 27°C, ~50% RH, and with a 17:7 (L:D) photoperiod, chosen to maintain the beetles physiologically in a reproductive state (Velarde et al., 2002). Light was provided by four 40-W fluorescent tubes about 1 m above the collectors.

Traps were changed each morning, eluting the traps with 400 µl methylene chloride. The beetles were transferred to clean collectors with fresh foliage, and the collection process was restarted. Maintenance was done daily because amounts of the target compound decreased if foliage was not fresh, and the chemical "background^ in the samples increased when the glassware was not cleaned frequently. This procedure was repeated as long as the beetles survived (usually > 30 d). Each collection was analyzed by gas chromatography–mass spectrometry (GC–MS) and then stored in a freezer (−70°C) until it was processed further.

For *G. pusilla*, the procedure in place during 2003 (when the initial collections were made from this species) employed groups of up to 50 males or females held in glass tubes of ca. 8 cm diam and 100 cm length, and the foliage amounts were greater. However, the 2005 collections from *G. pusilla* used the optimum procedure described above for *G. calmariensis*.

Electrophysiology. Samples from feeding male or female beetles of both species were analyzed by GC–EAD. The effluent from the GC column was split to a flame ionization detector (FID) and to the antenna of a male or female beetle. A glass pipette Ag/AgCl grounding electrode was inserted into the back of an excised beetle head. A second pipette serving as the recording electrode was placed in contact with the distal end of one antenna. Both pipettes were filled with physiological saline. GC and EAD data were analyzed by Syntech (Hilversam, the Netherlands) software, using previously described methods and equipment (Cossé and Bartelt, 2000).

Gas Chromatography–Mass Spectrometry. Coupled GC–MS was used to analyze all extracts (EI mode, 70 eV), using a Hewlett Packard 5973 mass selective detector, interfaced to an HP 6890 GC, controlled with Agilent ChemStation software (Release C.00.00), and with the Wiley spectral library with 275,000 spectra. A DB-1 column (30 m length, 0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) was used, programmed from 50°C/1 min, 10°C/min to 280°C, hold 5 min at 280°C. Other column types were also used. Injections were made in splitless mode, with He carrier gas and inlet and transfer line temperatures of 280°C. Positive chemical ionization (CI) mass spectra were acquired on the same instrument, with either methane or isobutane reagent gas. The high-resolution EI mass spectrum was obtained at the University of Illinois on a VG 70SE instrument with a GC inlet.

Gas Chromatography. Selected samples were analyzed by GC to estimate amounts of material present, using an HP 5890 Series II instrument with FID and column and parameters similar to those used for GC–MS. Quantitation was by the external standard method, relative to alkanes.

Preparation of NMR Sample by HPLC. The *G. calmariensis* compound was purified in one step by HPLC, using a Waters 515 pump with a Supelcosil LC-SI silica column (25 cm by 0.46 cm ID, 5 μm particle size), eluting with 5% ethyl ether (redistilled) in hexane (1 ml/min). The LDC/Milton Roy Spectro-Monitor D variable-wavelength UV detector was operated at 229 nm and was interfaced to an HP 3396 Series III integrator. Volatile collections were combined and concentrated under a stream of argon (three batches, each concentrated to about 500 μl). HPLC injections were 100 μl. The target compound eluted between 10 and 11 min and was essentially free of impurities, by GC–MS. Finally, the combined fractions were taken to dryness under a stream of nitrogen, and the compound was quickly taken up in 200 μl deuterobenzene. The sample for NMR contained 17 μg.

NMR Spectroscopy. NMR spectra were acquired at NCAUR on a Bruker Avance 500-MHz instrument with a 5-mm inverse broadband probe with a Z-gradient. Samples were run in a Bruker Microbore capillary-end tube (Wilmad-Labglass, Buena, NJ, USA #520-1A). The experiments conducted included 1D ^{1}H NMR and 2D ^{1}H COSY and HMBC.

Additional NMR spectra were acquired on this sample at Pfizer on a Bruker 500-MHz instrument equipped with a Bruker Dual CryoProbe™. The sample was transferred to a Shigemi tube, and 1D ^{1}H NMR, ^{13}C proton decoupled, DEPT135, and DEPT90 experiments and the 2D HMBC, HSQC, and HSQC-TOCSY experiments were conducted.

UV Spectroscopy. The UV spectrum was obtained on an HP 1040A diode array detector, using the Waters HPLC system described above. A quantitated sample was also run in a 1-cm cuvette on a Shimadzu UV-1601PC spectrophotometer to measure the extinction coefficient.

Microchemical Tests. Hydrogenation was conducted over Adam's catalyst (PtO_2) and over 10% palladium on carbon. Samples were dissolved in methylene chloride (ca. 1 μg in 100 μl) in a conical vial, and a small amount of catalyst was added (1 mg or less, barely visible in the vial). Hydrogen was bubbled through the solution from a fine needle for 5 min at room temperature, and then the sample was analyzed by GC–MS.

An aliquot was also reduced with $LiAlH_4$. The reagent (1 M in ether, Aldrich Chemical Co., Milwaukee, WI, USA) was diluted further with dry ether, and a drop was added to a sample of the beetle compound (ca. 500 ng) in ether. After several minutes, the reaction was quenched with a minimal amount of water and analyzed by GC–MS. Treatment with diazomethane was done essentially as described by Levitt (1960) on the beetle compound and on the standards, heptanoic acid and γ-decalactone.

Synthesis. The synthetic scheme is summarized in Figure 1. Briefly, the strategy was to prepare 3,4-dimethylfuran (**7c**), construct hydroxyl and acyl side chains at the 2 and 5 positions, and then close the lactone ring.

To make 3,4-dimethylfuran, 3,4-bis-(acetyloxymethyl)furan **6** was first prepared by a Diels-Alder reaction between butynediol diacetate **3** (obtained by acetylating diol **2**) and 4-phenyloxazole **5** (made from phenacyl bromide **4** and ammonium formate). The Diels–Alder adduct decomposed spontaneously to **6** and benzonitrile. All of the above reactions were done as described by Hutton et al. (1979). Diacetate **6** was converted to diol **7a** by transesterification with MeOH/MeONa (Christie, 1993). Diol **7a** was converted to dichloride **7b** with thionyl chloride, which was reduced to 3,4-dimethylfuran **7c** with $LiAlH_4$ (see Rawson et al., 1979, for both of these reactions).

The precursor for the 5-carbon side chain was prepared by converting 1,5-pentanediol **8** to iodohydrin **9a** by refluxing with HI in a two-phase system (Kang et al., 1985). The hydroxyl group of **9a** was protected as the tetrahydropyranyl (THP) ether **9b** (Miyashita et al., 1977). Furan **7c** was smoothly alkylated with **9b** after the 2 position was lithiated with butyllithium, giving trisubstituted furan **10** (Nolan and Cohen, 1981). To prepare the 3-carbon acyl side chain, **10** was first carbonylated to **11** by lithiation of the 5 position, followed by reaction with *N*,*N*-dimethylformamide (Koenig, 2002). Aldehyde

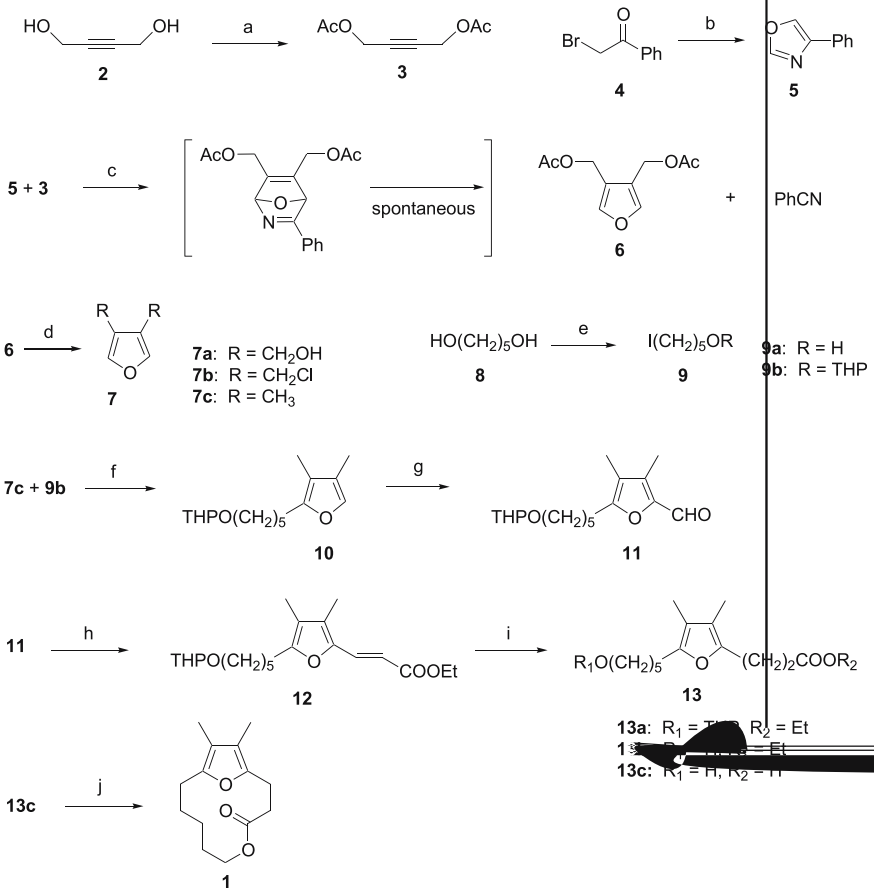


FIG. 1. Synthesis of *Galerucella* furan lactone (**1**). (a) Ac_2O, PTSA, 110–115°C, 3 hr; quant.; (b) ammonium formate, isobutyl formate, formic acid, azeotropic removal of water at 92–94°C, 7 hr; 51%; (c) Na_2CO_3, 205–206°C, 24 hr; 64%; (d) (*i*) NaOMe (0.2 equiv.), MeOH, rt, 1 hr; quant.; (*ii*) $SOCl_2$, pyridine, CH_2Cl_2, −5°C, 15 min; 60%; (*iii*) $LiAlH_4$, ether, addition at 0°C, then reflux, 4 hr; 70%; (e) (*i*) concentrated HI, hexane (two phases), reflux, 4 hr; 35%; (*ii*) dihydropyran, pyridinium *p*-toluenesulfonate (PPTS), CH_2Cl_2, rt, 4 hr; 86%; (f) addition of BuLi to **7c**, THF, −15°C, 2 hr, followed by addition of **9b**, −15°C, 1 hr, then rt, 1 hr; 62%; (g) BuLi, THF, 0°C, 2 hr, then dimethylformamide, 0°C, 1 hr; 40%; (h) triethyl 2-phosphonoacetate, BuLi, THF, 0°C, 45 min; 40%; (i) (*i*) 10% Pd on carbon, hexane, H_2 (1 atm), rt, 5 min; 95%; (*ii*) PPTS, EtOH, 55°C, 2.5 hr; quant.; (*iii*) KOH, 1:1 MeOH/H_2O, 45°C, 2 hr, by GC reaction complete; (j) 2,4,6-trichlorobenzoyl chloride, Et_3N, THF, rt, 2 hr, then 4-(*N*,*N*-dimethylamino)pyridine, toluene, reflux, addition over 3.5 hr; 75%.

11 was elaborated to unsaturated ester **12** by a Wittig–Horner condensation with triethyl 2-phosphonoacetate (Boutagy and Thomas, 1974). The side-chain double bond was selectively reduced by hydrogenation over 10% Pd on carbon in hexane to give **13a** (Lie Ken Jie, 1981).

Compound **1** was completed by deprotecting the hydroxyl (Miyashita et al., 1977, giving **13b**) and acyl functions (KOH in aqueous MeOH) and closing the ring. Hydroxy acid **13c** was activated by conversion to the mixed anhydride with 2,4,6-trichlorobenzoyl chloride, followed by cyclization under high-dilution conditions using 4-(*N*,*N*-dimethylamino)pyridine as the catalyst (Sinha et al., 1993). Purification on silica gel (10% ether in hexane, after 5% ether in hexane) yielded 30 mg of **1** (91% pure).

The synthesis is described in detail in the online supplementary information available for this article, at http://dx.doi.org/10.1007/s10886-005-9026-3 and is accessible for authorized users.

Field Lures. Red rubber septa (Aldrich) were cleaned by Soxhlet extraction with CH_2Cl_2, and loaded with synthetic **1** (500 μg in 100 μl of hexane). Loaded septa were stored at −20°C until used. One freshly prepared septum was placed into a laboratory volatiles-collection apparatus to determine the emission rate of **1**. Volatiles were collected continuously for 31 d and were quantitated every 1–4 d by GC, using heptadecane as internal standard.

Field Tests. Field tests of synthetic **1** were conducted on May 10, 17, and 18, 2005, in two wetlands in northeastern Illinois (Cook County) known to have infestations of purple loosestrife and populations of *Galerucella* spp. The field sites were at Hyde Lake (41°39.9′N, 87°33.1′W) and Powderhorn Lake (41°38.6′N, 87°32.0′W). The three test days were mostly sunny, with maximum temperatures of ca. 20°C. Beetles were observed on plants at both locations, but the season was slightly more advanced at Hyde Lake: Eggs were easily found at Hyde Lake on May 17, but none were apparent at Powderhorn Lake on that date.

Traps were 15 × 15-cm yellow sticky cards with adhesive on both sides ("Sticky Strips,^ Olson Products, Medina, OH, USA, cut in half). These were attached near the tops of bamboo stakes (1.2 m in length). Lures were fastened to the tops of the sticky cards with wire.

Experiments were organized as paired *t* tests, with a treated and a control trap in each pair. Traps were placed near purple loosestrife, but not necessarily in dense stands. Trap positions of a pair were separated by 3–4 m and were chosen to be as similar as possible with respect to height, density, and type of vegetation. Assignment of treatments within trap pairs was randomized. Spacing between pairs was at least 10 m. Overall, 15 pairs of traps were run at Hyde Lake (on May 10 and 17), and 20 pairs were run at Powderhorn Lake (on May 17 and 18).

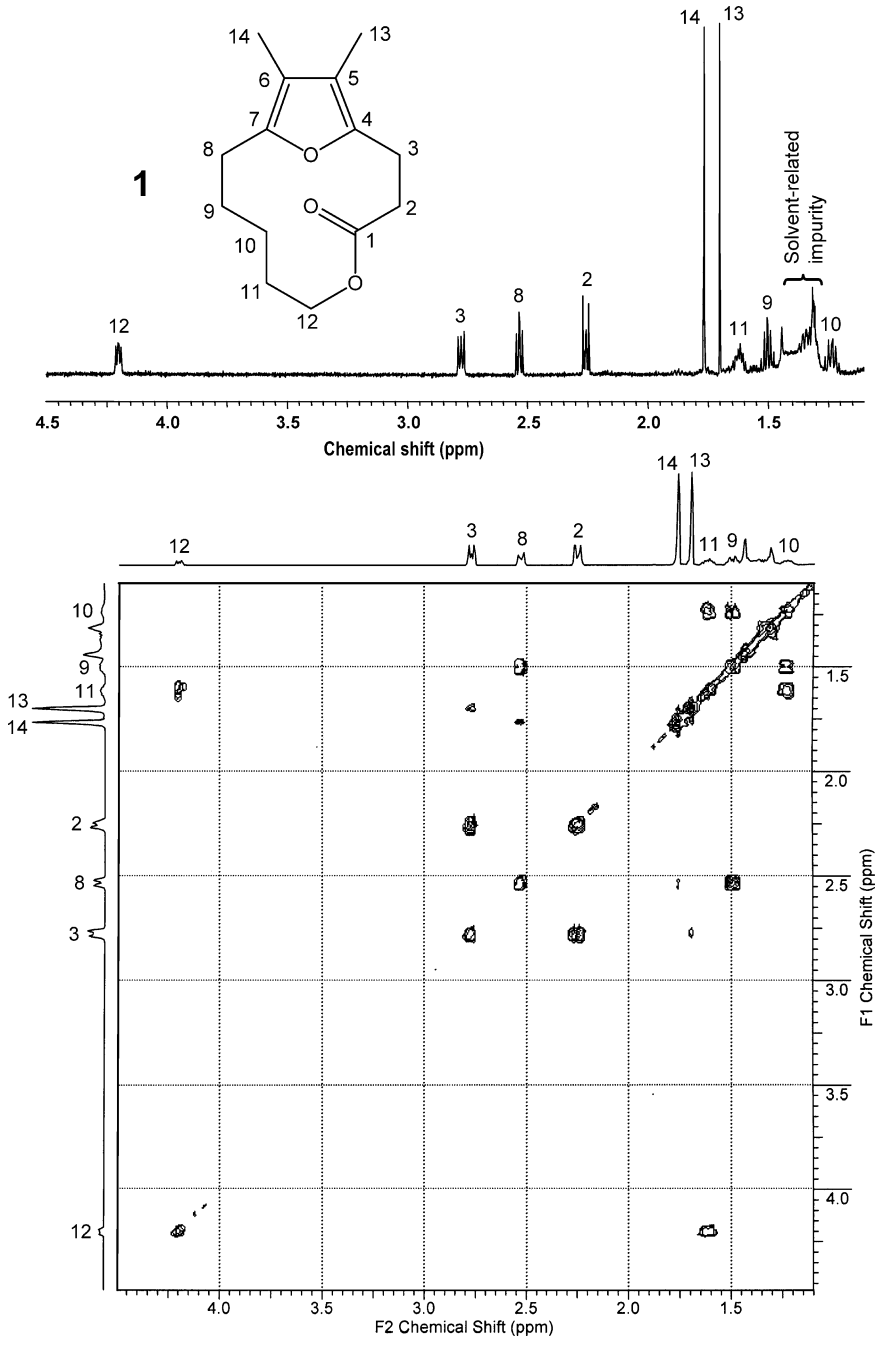
Traps were deployed in midmorning, before beetles became active, and the experiment was terminated between 3:00 PM and 5:00 PM. Beetle numbers were recorded at the end of the experiment, and all specimens were examined under the microscope for tibial spurs. Samples of males (five of each species from each field site) were dissected to examine genitalia (Manguin et al., 1993).

RESULTS AND DISCUSSION

Identification and Sexing of Beetles. As was found previously with *G. calmariensis* (Cossé, 2004), the males of *G. pusilla*, but not the females, have spurs on their mesotibiae. However, analogous spurs were not found on the metatibiae of male *G. pusilla*, as they had been on males of *G. calmariensis*. These characters allowed males of the two species to be distinguished from each other and from females. Moreover, these determinations could be done without causing injury (compare to Manguin et al., 1993). Species determinations made using the spur characters were subsequently verified by examination of genitalia at NCAUR and by submission to experts at other institutions. We were unable to confidently separate females of *G. calmariensis* and *G. pusilla* from mixed populations using the criteria of Manguin et al. (1993).

Male-Specific Compound. GC–MS comparison of volatiles from feeding male and female *G. calmariensis*, supplemented by GC–EAD analysis, revealed a likely pheromone. Figure 2 (top) shows example GC traces from feeding males and females. Most of the peaks in these samples were from the plant material. However, one compound was found only from males, and it elicited an intense response from the antennae of both sexes (lower part of Figure 2). The amount of the male-specific compound depended on the conditions during collections and tended to be low early in the project when techniques were still evolving. Some early samples that elicited the characteristic EAD response had such a small amount of the male-specific compound that it was not detected by GC–FID. As the beetle-handling and volatile-collecting procedures were improved, the compound became more prominent in chromatograms (e.g., Figure 2), and it amounted to as much as 5–10% of the total GC peak area in some samples. Males produced a maximum 30–50 ng per male per day. Production typically began within a week of setting up collectors and then continued for a month or more. In the final collection effort during 2004, the compound was detected in 69 of the 81 samples, and all of those not showing it were from the initial 9 days. There was no evidence for other sex-specific compounds that elicited antennal responses.

For *G. pusilla*, 36 of the 50 collections from males in 2003 had detectable amounts of the same compound, but none was found in 21 collections from


1
14
13
6
5
7
4
8
3
O
9
O
2
10
1
11
O
12
Solvent-related impurity
Chemical shift (ppm)
F2 Chemical Shift (ppm)
F1 Chemical Shift (ppm)

The beetle-derived compound had a UV maximum at 229 nm (extinction coefficient 7000), suggesting that there were two double bonds in conjugation but not three.

Other microchemical reactions and liquid chromatography results gave useful structural information. The beetle compound reacted with $LiAlH_4$, indicating that a carbonyl or other reducible group was present, although the product from this reaction was not recovered. The compound did not react with diazomethane, indicating no acidic protons. The polarity of the beetle compound on silica (HPLC) was lower than would be expected if a free hydroxyl group were present; the compound eluted with 5% ether in hexane, rather than requiring 25–50% ether in hexane, typically needed to elute an alcohol.

NMR Spectroscopy. NMR analysis was conducted on the purified compound (17 *2*g). The 1D proton spectrum is shown in Figure 4 (top), and the proton shifts and coupling information are summarized in Table 1. The proton spectrum accounted for all 20 of the protons expected from the high-resolution mass spectrum. The most downfield resonance (signal 12, two protons) suggested a methylene attached to an oxygen, and three midfield signals (2, 3, and 8, all with two protons) suggested methylenes adjacent to unsaturated carbons. The methyl groups (13 and 14) had shifts suggesting attachment to unsaturated carbons. The three remaining resonances (9, 10, and 11, each with two protons) were consistent with methylenes attached only to saturated carbons. Importantly, there was no evidence for olefinic protons.

The COSY spectrum (Figure 4, bottom) indicated two spin systems that were isolated from each other, consisting of two and five methylene units (signals 2 and 3 and 8–12, respectively). In addition, there was evidence for long-range, homoallylic coupling of the methyl groups into the main spin systems (specifically, between methyl 13 and methylene 3 and between methyl 14 and methylene 8). The methyl signals were broadened by this coupling but were not obviously split.

There were two other interesting features of the ^1H data. First, both protons of each methylene pair apparently had the same chemical shift, which would be extremely unlikely if one or more asymmetric centers were present in the molecule. Second, four of the methylenes (2, 3, 8, and 12) were nominally triplets but had second-order character (appearing as aa′bb′ multiplets). This was probably due to restricted rotation, perhaps because of a ring structure.

FIG. 4 Proton NMR spectrum of the male-specific compound from *G. calmariensis* and assigned structure (top) and the proton COSY spectrum for the compound (bottom). A solvent-related impurity is evident in the proton spectrum; it does not correlate to any of the numbered signals in the COSY spectrum, indicating that it is unrelated to the beetle compound.

TABLE 1. SUMMARY OF ^{1}H AND ^{13}C NMR DATA FOR THE MALE-SPECIFIC *Galerucella* COMPOUND

Carbon position	^{1}H: shift (multiplicity), J	^{13}C: shift,[a] (multiplicity)	2D $^{13}C/^{1}H$ (protons observed)	
			HMBC	HSQC–TOCSY[b]
1	–	172.0,[c] (C)	2, 3, 12	
2	2.26 (2nd order)[d]	34.36 (CH_2)	3	2, 3
3	2.78 (2nd order)[d]	22.38 (CH_2)	2	2, 3
4	–	146.1,[c] (C)	2, 3, 13	
5	–	114.4,[c] (C)	13, 14	
6	–	114.4,[c] (C)	8,[e] 13, 14	
7	–	147.6,[c] (C)	8, 9, 14	
8	2.53 (t with 2nd order character), $J_{8,9} \sim 6.5$ Hz	22.98 (CH_2)	9	8, 9
9	1.50 (p), $J_{8,9} \sim J_{9,10} \sim 6.5$ Hz	24.73 (CH_2)	8	8, 9
10	1.24 (p), $J_{9,10} \sim J_{10,11} \sim 6.5$ Hz	21.86 (CH_2)	8, 9, 12	9, 10, 11
11	1.62 (m)	24.95 (CH_2)	9	10, 11, 12
12	4.20 (2nd order)[d]	62.35 (CH_2)		11, 12
13	1.70 (s), $J_{3,13} < 1$ Hz	7.50 (CH_3)		13
14	1.77 (s), $J_{8,14} < 1$ Hz	7.40 (CH_3)		14

[a] Unless otherwise indicated, carbon shifts were observed directly and numbers of attached protons were confirmed by DEPT135 experiment; associations between particular carbons and protons based on HSQC experiment.
[b] Mixing time was 17 msec; the protonated carbons were correlated to their own protons and also to protons on immediately adjacent carbons.
[c] Shifts and assignments determined from HMBC experiment.
[d] Apparent aa′bb′ systems; coupling constants are not directly observable.
[e] Redundant carbon shifts (δ 114.4) make assignment of cross peak ambiguous.

At this point, the chains of two and five methylenes and the two relatively isolated methyls accounted for 9 of the 14 carbons indicated by high-resolution mass spectrometry (HRMS), but there was little information as to how these protonated groups were connected through the five remaining, unprotonated carbons. The 2D heteronuclear multiple bond correlation (HMBC) experiment (for observing 2- and 3-bond proton/carbon correlations) gave the needed information.

Because of the small sample size, the NCAUR instrument showed only the most intense HMBC cross peaks, those related to the methyl group protons, but these were informative. Methyl protons 13 (δ 1.70) were correlated to carbon resonances at δ 114.4 and 146.1, and methyl protons 14 (δ 1.77), to carbon resonances at δ 114.4 and 147.6. Because three cross peaks were anticipated for each methyl, it was postulated that there were really two carbons at δ 114.4 and that both of these were coupled to both of the methyl

groups. The furan ring in **1** would meet these criteria, and the observed carbon shifts were consistent with a furan (Glass et al., 1975; Levy et al., 1980). The furan structure also accounted for the two double bonds revealed by hydrogenation. Tetrasubstituted furans have been shown to be reactive to catalytic hydrogenation (Glass et al., 1975). Furthermore, a substituted furan could explain the observed UV maximum at 229 nm (Scott, 1964; Glass et al., 1975). The ring of the furan would also account for one of the three remaining, unexplained degrees of unsaturation.

The initial HMBC result was confirmed on the more sensitive instrument at Pfizer, and additional information was obtained as well. Nearly all of the possible HMBC correlations for **1** involving the unprotonated carbons were observed, as were many of those involving the protonated carbons (summarized in Table 1). The experiment showed the attachment of the methylene chains to the furan ring and also gave evidence for the lactone function. In particular, carbon 1 was attached to methylene 2 and, through an oxygen, to methylene 12, and the shift of carbon 1 (δ 172.0) was consistent with a lactone carbonyl (Levy et al., 1980). The lactone group accounted for the two remaining degrees of unsaturation. Other evidence supporting the lactone was the reactivity with $LiAlH_4$ (ester reduction), failure to react with diazomethane (no free carboxyl), and the relatively low polarity by silica gel chromatography.

The ^{13}C spectrum acquired at Pfizer (30,000 scans) showed peaks for all of the protonated carbons (shifts summarized in Table 1). The DEPT135 experiment confirmed which carbons were methyls and which were methylenes. No peaks were observed in the DEPT90 experiment, consistent with no methine protons. The HSQC experiment showed all of the possible one-bond proton/carbon correlations, and these were used to assign the carbon shifts to the particular positions listed in Table 1. The HSQC–TOCSY experiment (Table 1) supported the proton/carbon and proton/proton correlations obtained from the HSQC and COSY experiments, respectively. Taken together, the data fully determined the structure of the beetle compound **1**, 12,13-dimethyl-5,14-dioxabicyclo[9.2.1]tetradeca-1(13),11-dien-4-one. The beetle-derived and synthetic **1** were identical with respect to GC retention, mass spectrum, proton NMR spectrum, and GC–EAD analysis (males and females of both species).

Septum Emissions. Over a period of 31 d, the mean emission rate of **1** from a septum treated with 500 μg of this compound was 30 (± 7 SD) ng/hr ($N = 17$). The rate remained stable over the 31-d period (linear regression of release rate over time not significant, $t = -1.67$, $P = 0.12$). Calculated from collected amounts and septum load, less than 5% of the applied compound actually volatilized during the test. The release rate was approximately 15 times the maximum observed daily average per live male in the laboratory. However, if the beetles only produce pheromone during certain periods of the day (rather than throughout the day), or if not all of the males in the aeration tubes were

actually emitting, or both, then the peak emission rate from males could approach or exceed the release rate from septa.

Field Tests. Traps baited with synthetic **1** caught more beetles (10.3 ± 9.9 SD) than controls (1.8 ± 2.6; $N = 35$, $t = 10.3$, $df = 34$, $P < 0.001$; paired t test after $\log(X + 1)$ transformation). The control catch never exceeded that of the treated trap for any pair, although no beetles were caught in 2 of the 35 blocks.

Examination of the trapped beetles gave the results summarized in Table 2. Species determinations based on tibial spurs were fully supported by dissections of genitalia. By a chi-square test, the overall difference between the pheromone and the control was again highly significant ($P < 0.001$), but the treatment by beetle-category interaction was not significant ($P = 0.16$). Thus, the effect of the pheromone, expressed as the percentage of the catch found in pheromone traps, was reasonably consistent over all six beetle categories (involving species, sexes, and locations).

The overall captures of males (Table 2, totals for pheromone plus control) suggested that *G. calmariensis* is the minor species at the Hyde Lake site (24% of the captured males) but is the major species at Powderhorn Lake (88% of the captured males). Based on a 2 by 2 contingency table with the trap totals for males, these percentages of *G. calmariensis* at the two locations were

TABLE 2. SUMMARY OF *Galerucella* TRAPPING RESULTS, CHICAGO, IL, MAY 2005 (DATA ARE TOTALS OVER REPLICATIONS FOR INDIVIDUAL TREATMENTS, BEETLE CATEGORIES, AND LOCATIONS)

Beetle category	Trap catch			Percent on pheromone
	Pheromone	Control	Total (pheromone + control)	
Hyde Lake (15 replications)				
Male *G. calmariensis*	25	8	33	76
Male *G. pusilla*	86	21	107	80
Female *Galerucella* spp.	150	25	175	86
Powderhorn Lake (20 replications)				
Male *G. calmariensis*	51	8	59	86
Male *G. pusilla*	7	1	8	88
Female *Galerucella* spp.	33	1	34	97
Overall totals (35 replications)	352	64	416	85

Chi-square tests: (1) Using the two overall totals for pheromone and control, test of pheromone effect (H_0: Pheromone and control trap catches are equal): $\chi^2 = 199$, 1 *df*, $P < 0.001$. (2) Using the 12 treatment totals for the individual beetle categories (6 for pheromone and 6 for control), test of treatment by beetle–category interaction (H_0: Pheromone trap catch, as % of total, is the same for all beetle categories): $\chi^2 = 7.88$, 5 *df*, $P = 0.16$.

significantly different (χ^2 = 76.3, 1 *df*, $P < 0.001$). Given that substantial numbers of females were caught in both places, we suggest that females of both species were caught during the study, although the actual numbers of each were not determined. An important question for future research would be whether trap catches reflect actual species compositions around the trap sites. Establishing a correlation of this sort would enhance the value of the pheromone as a monitoring tool. A final pattern in the trap data was that females represented a significantly smaller percentage of the trap catch at Powderhorn Lake (34%) than at Hyde Lake (56%) (χ^2 = 14.7, 1 *df*, $P < 0.001$). The reason for this difference is unknown, but may reflect the slightly later phenology at Powderhorn Lake.

Biological Activity and Future Research. A surprising finding was that both *G. calmariensis* and *G. pusilla* produce and respond to the same compound. The two species often occur at the same location and at the same time of year in their native Europe (Manguin et al., 1993). It was suggested by Manguin et al. (1993) that the species might have different pheromones, but the evidence so far is the opposite. It is unclear how interspecific competition/confusion is avoided, whether there are subtle differences in diel activity periods of each species, additional pheromone components that have yet to be discovered, or still other mechanisms for long-range species recognition and reproductive isolation.

Related Compounds from the Literature. Several other furan-containing fatty acids derivatives that are structurally related to pheromone **1** have been reported. The example shown in Figure 5 is found in minor amounts in grasses,

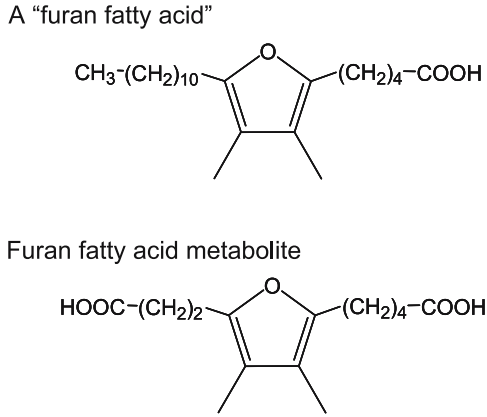


FIG. 5. Natural compounds chemically related to **1**: A “furan fatty acid^ and the product obtained from it after metabolism by a rat (see text).

potato leaves, and birch tree leaves (Hannemann et al., 1989) and in olive oil (Boselli et al., 2000). The same compound is also a major lipid in the testes of a fish, the northern pike (*Esox lucius* L.) (Glass et al., 1975). Related lipids in these and other organisms have different numbers of methylenes in the side chains, unsaturation in a side chain, and/or have one of the ring methyls replaced by hydrogen. When fed to rats, furan fatty acids were partially metabolized and excreted in the urine; one such example is shown in Figure 5 (Sand et al., 1983). Interestingly, this metabolite differs from **1** only in the oxidation state at the end of the 5-carbon side group and in the absence of a closed lactone ring.

Other insects with macrocyclic lactone pheromones are known. Examples include the beetle *Oryzaephilus surinamensis* (L.) (Pierce et al., 1985), the beetle *Cryptolestes ferrugineus* (Stephens) (Wong et al., 1983), and the true bug *Piezodorus hybneri* (Gmelin) (Leal et al., 1998). Insect macrolides have a range of ring sizes, may have double bonds or branches, and may be chiral or achiral. However, none was previously found that includes the furan structure. Compound **1** is unique as a natural product and further extends the known chemical diversity of pheromones within the Chrysomelidae.

Acknowledgments—Dr. Luke Skinner, Minnesota Department of Natural Resources, St. Paul, MN, and Dr. A. S. Konstantinov of the USDA-ARS Systematic Entomology Laboratory, Beltsville, MD, verified the species identifications. Dr. Skinner also provided *G. pusilla* adults used for collection of volatiles and GC–EAD studies in 2003 and 2005. We are grateful to Professor James Gloer, Department of Chemistry, University of Iowa, for helpful discussions about NMR techniques and possible structures. We also thank Dr. James Oliver, USDA-ARS, Beltsville, MD, for thoughts about structures and future syntheses. The high-resolution mass spectrum was acquired by Mr. Furong Sun, Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, Champaign, IL.

REFERENCES

BARTELT, R. J. and CHIANG, H. C. 1977. Field studies involving the sex-attractant pheromones of the western and northern corn rootworm beetles. *Environ. Entomol.* 6:853–861.

BARTELT, R. J., COSSÉ, A. A., ZILKOWSKI, B. W., WEISLEDER, D., and MOMANY, F. A. 2001. Male-specific sesquiterpenes from *Phyllotreta* and *Aphthona* flea beetles. *J. Chem. Ecol.* 27:2397–2423.

BARTELT, R. J., WEISLEDER, D., and MOMANY, F. A. 2003. Total synthesis of himachalene sesquiterpenes of *Aphthona* and *Phyllotreta* flea beetles. *Synthesis* 2003:117–123.

BOSELLI, E., GROB, K., and LERCKER, G. 2000. Determination of furan fatty acids in extra virgin olive oil. *J. Agric. Food Chem.* 48:2868–2873.

BOUTAGY, J. and THOMAS, R. 1974. Olefin synthesis with organic phosphonate carbanions. *Chem. Rev.* 74:87–99.

CHRISTIE, W. W. 1993. Preparation of ester derivatives of fatty acids for chromatographic analysis. *Adv. Lipid Meth.* 2:69–111.

COSSÉ, A. A. 2004. Presence of tibial spurs as a male sexual character for *Galerucella calmariensis* (Coleoptera: Chrysomelidae). *J. Entomol. Sci.* 39:281–283.

COSSÉ, A. A. and BARTELT, R. J. 2000. Male-produced aggregation pheromone of *Colopterus truncatus*: Structure, electrophysiological and behavioral activity. *J. Chem. Ecol.* 26:1735–1748.

COSSÉ, A. A., BARTELT, R. J., and ZILKOWSKI, B. W. 2002. Identification and electrophysiological activity of a novel hydroxy ketone emitted by male cereal leaf beetles. *J. Nat. Prod.* 65:1156–1160.

COSSÉ, A. A., BARTELT, R. J., ZILKOWSKI, B. W., BEAN, D. W., and PETROSKI, R. J. 2005. The aggregation pheromone of *Diorhabda elongata*, a biological control agent of saltcedar (*Tamarix* spp.): Identification of two behaviorally active components. *J. Chem. Ecol.* 31:669–682.

DICKENS, J. C., OLIVER, J. E., HOLLISTER, B., DAVIS, J. C., and KLUN, J. A. 2002. Breaking a paradigm: Male-produced aggregation pheromone for the Colorado potato beetle. *J. Exp. Biol.* 205:1925–1933.

GLASS, R. L., KRICK, T. P., SAND, D. M., RAHN, C. H., and SCHLENK, H. 1975. Furanoid fatty acids from fish lipids. *Lipids* 10:695–702.

GREVSTAD, F. S. and HERZIG, A. L. 1997. Quantifying the effects of distance and conspecifics on colonization: Experiments and models using the loosestrife leaf beetle, *Galerucella calmariensis*. *Oecologia* 110:60–68.

HANNEMANN, K., PUCHTA, V., SIMON, E., ZIEGLER, H., ZIEGLER, G., and SPITELLER, G. 1989. The common occurrence of furan fatty acids in plants. *Lipids* 24:296–298.

HIGHT, S. D., BLOSSEY, B., LAING, J., and DECLERCK-FLOATE, R. 1995. Establishment of insect biological control agents from Europe against *Lythrum salicaria* in North America. *Environ. Entomol.* 24:967–977.

HUTTON, J., POTTS, B., and SOUTHERN, P. F. 1979. Synthesis of 3,4-bis-acetoxymethylfuran. *Synth. Commun.* 9:789–797.

KANG, S.-K., KIM, W.-S., and MOON, B. H. 1985. An effective method for the preparation of omega-bromoalkanols from alpha, omega-diols. *Synthesis* 1985:1161–1162.

KOENIG, B. 2002. Product class 9: Furans. *Sci. Synth.* 9:183–285.

KRYSAN, J. L., MCDONALD, I. C., and TUMLINSON, J. H. 1989. Phenogram based on allozymes and its relationship to classical biosystematics and pheromone structure among eleven Diabroticites (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 82:574–581.

LEAL, W. S., KUWAHARA, S., SHI, X., HIGUCHI, H., MARINO, C. E. B., ONO, M., and MEINWALD, J. 1998. Male-released sex pheromone of the stink bug *Piezodorus hybneri*. *J. Chem. Ecol.* 11:1817–1829.

LEVY, G. C., LICHTER, R. L., and NELSON, G. L. 1980. Carbon-13 Nuclear Magnetic Resonance Spectroscopy, 2nd Edition. John Wiley and Sons, New York.

LEVITT, M. J. 1960. Rapid methylation of micro amounts of nonvolatile acids. *Anal. Chem.* 45:618–620.

LIE KEN JIE, M. S. F. 1981. 32. Chemical synthesis of furanoid fatty acids. *Meth. Enzymol.* 72:443–471.

MANGUIN, S., WHITE, R., BLOSSEY, B., and HIGHT, S. D. 1993. Genetics, taxonomy, and ecology of certain species of *Galerucella* (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 86:397–410.

MUTO, S., BANDO, M., and MORI, K. 2004. Synthesis and stereochemistry of the four himachalene-type sesquiterpenes isolated from the flea beetle (*Aphthona flava*) as pheromone candidates. *Eur. J. Org. Chem.* 2004:1946–1952.

MIYASHITA, N., YOSHIKOSHI, A., and GRIECO, P. A. 1977. Pyridinium *p*-toluenesulfonate: A mild and efficient catalyst for the tetrahydropyranylation of alcohols. *J. Org. Chem.* 42:3772–3774.

NOLAN, S. M. and COHEN, T. 1981. A general approach to 4-substitution of 2-alkyl alkylfurans. *J. Org. Chem.* 46:2473–2476.

PIERCE, A. M., PIERCE, H. D. JR., OEHLSCHLAGER, A. C., and BORDEN, J. H. 1985. Macrolide aggregation pheromones in *Oryzaephilus surinamensis* and *O. mercator* (Coleoptera: Cucujidae). *J. Agric. Food Chem.* 33:848–852.

RAO, S., COSSÉ, A. A., ZILKOWSKI, B. W., and BARTELT, R. J. 2003. Aggregation pheromone of the cereal leaf beetle: Field evaluation and emission from males in the laboratory. *J. Chem. Ecol.* 29:2165–2175.

RAWSON, D. I., CARPENTER, B. K., and HOFFMAN, H. M. R. 1979. Loss of allyl cation configuration in cycloadditions to electron-rich conjugated dienes. *J. Am. Chem. Soc.* 101:1786–1793.

SAND, D. M., SCHLENK, H., THOMA, H., and SPITELLER, G. 1983. Catabolism of fish furan fatty acids to urofuran acids in the rat. *Biochim. Biophys. Acta* 751:455–561.

SCOTT, A. I. 1964. Interpretation of the Ultraviolet Spectra of Natural Products. MacMillan, New York.

SINHA, S., SINHA-BAGCHI, A., and KEINAN, E. 1993. A general approach to enantiomerically pure methylcarbinols: Asymmetric synthesis of antibiotic (−)-A26771B and the WCR sex pheromone. *J. Org. Chem.* 58:7789–7796.

SOROKA, J. J., BARTELT, R. J., ZILKOWSKI, B. W., and COSSÉ, A. A. 2005. Male-produced aggregation pheromone of *Phyllotreta cruciferae*: Behavioral responses of flea beetles in the field. *J. Chem. Ecol.* 31:1829–1843.

VELARDE, R. A. M., WIEDENMANN, R. N., and VOEGTLIN, D. J. 2002. Influence of photoperiod on the overwintering induction of *Galerucella calmariensis* L. *Biocontrol* 47:587–601.

WONG, J. W., VERIGIN, V., OEHLSCHLAGER, A. C., BORDEN, J. H., PIERCE, H. D. JR., PIERCE, A. M., and CHONG, L. 1983. Isolation and identification of two macrolide pheromones from the frass of *Cryptolestes ferrugineus*. *J. Chem. Ecol.* 9:451–474.